In the name of God

Aptamer-based liposomes improve specific drug loading and release

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Abstract:

- Aptamer technology has shown much promise in cancer therapeutics for its targeting abilities
- In this study, drug-binding aptamers employed to actively load drugs into liposomes
- A series of DNA aptamer sequences specific to doxorubicin designed, displaying multiple binding sites and various binding affinities
- Optimization of the charge and drug/aptamer ratios resulted in ≥80% encapsulation efficiency of doxorubicin

- Release and therapeutic efficacy of liposomal doxorubicin could be controlled by the aptamer's structure
- The aptamer exhibiting a specific intermediate affinity is the best suited to achieve high drug loading while maintaining efficient drug release and therapeutic activity
- This strategy was successfully applied to tobramycin, a hydrophilic drug suffering from low encapsulation into liposomes

Introduction:

- Aptamer technology, although discovered for 25 years, is still evolving
- Aptamers are RNA or DNA sequences generated to

exhibit high affinity and specificity against a broad range of targets

- Aptamers recognize their specific targets due to the unique three dimensional structure
- Nucleotides aptamers compared to antibodies:

  1) lower immunogenicity

  2) higher thermal stability

  3) rapid and large scale synthesis

  4)lower production costs

- The pioneering work of Farokhzad et al. first demonstrated the potential of conjugating an aptamer to the surface of polymeric nanoparticles for targeting prostate cancers in vivo
- Major limitation of nanocarriers: loading sufficient therapeutics into nanocarriers, while controlling its release rate
- Benefits of liposomes:

  1) large internal volume for high drug loading

  2)prolonged circulation times

  3) controlled biodistribution

  4) excellent biocompatibility and biodegradability

Doxil:

- Commercialized liposomes of doxorubicin
- Able to reach up to 10,000 molecules of doxorubicin per liposome
- The formulation significantly reduced the cardiotoxicity of doxorubicin
- The strong entrapment of the drug within the core significantly reduced its release & its therapeutic efficacy

- the loading and release rate of the drug from aptamer-drug complexes is a function of the sequence and the number of binding sites
- Disadvantages of aptamer- drug complexes:

  1) low stability in the blood

  2)limited drug loading capacity

  3) some inherent immunogenicity

- Incorporating the drug-aptamer complex into liposomes: improve specific drug loading and offer a better control over the release rate to improve the therapeutic efficiency

Materials & Methods:

- Dissociation constants of aptamer-doxorubicin complexes:

  - ✓ obtained by monitoring the quenching of doxorubicin fluorescence at various aptamer concentrations
  - ✓ Fluorescence emission spectrum(λex 485 nm, λem 520–700 nm) was recorded at 37 °C on a Cary Eclipse Fluorescence spectrophotometer

- Preparation and characterization of liposomes:
  - ✓ All liposome formulations, except Doxil-like, were prepared using the hydration method
  - ✓ Cationic liposomes: DOTAP, cholesterol and DSPE-PEG2000 (50/48/2 molar ratio)
  - ✓ “No cationic lipid” formulation: POPC, cholesterol and DSPEPEG2000 (55/40/5 molar ratio)
  - ✓ Doxil-like liposomes: 55% (DSPC), 40% cholesterol and 5% DSPEPEG2000
- Liposome hydrodynamic diameter and ζ-potential: Malvern Zetasizer Nano ZS

❖ Preparation of aptamer-loaded lipoplexes:

✓ Add Aptamer solution to liposomal solution (1:1 v/v) at N/P ratios (0.5–15)

✓ N: number of amines (molar quantity of DOTAP)

✓ P: is the number of phosphorous groups of aptamers (corresponding to the number of nucleotides)

❖ N/P ratio was optimized for each formulation to encapsulate>90% of aptamers

❖ Encapsulation efficiency of aptamers:

✓ Two methods to determine the encapsulation efficiency of aptamers into cationic liposomes:

1)Indirect method

2)Direct method

✓ Indirect method:

The residual aptamer concentration in solution was quantified using fluorescent intercalating probes SYBRGold or SYBRGreen for Apt-Ctrl-2 or all other aptamers, respectively.

each sample analyzed with a Safire microplate reader (λexc 496 nm, λem 523 nm for both SYBRGreen and SYBRGold).

Eq (1):

$$EE\ (\%) = \frac{\text{Total DNA} - \text{Free DNA}}{\text{Total DNA}} * 100\% \qquad (1)$$

✓ Direct method:

the amount of encapsulated aptamers within the lipoplexes was quantified directly according to a fluorescent Assay (496/523 nm (λexc/λem))

- ✓ t=30 s: lipoplexes added in the cuvette
- ✓ t = 100 s: Triton X100 was added to disrupt the liposomes
- ✓ t = 200 s: Final fluorescence was recorded

$$EE\ (\%) = 100 - \left(\frac{\text{Initial intensity}}{\text{Final intensity}}\right) * 100\% \qquad (2)$$

❖ Stability of lipoplexes:

- ✓ Encapsulation efficiency of aptamers was measured at 0, 1, 2, 6, 8, 12 and 24 h
- ✓ hydrodynamic diameter and polydispersity index were measured at days 0, 3, 5 and 7.

❖ Doxorubicin-loaded lipoplexes:

- ✓ Equal volumes of lipoplexes and doxorubicin solutions were combined at various doxorubicin/aptamer molar ratios (1:1–25:1)
- ✓ N/P ratio and drug/aptamer ratio were optimized for each formulation
- ✓ for Doxil-like liposomes, doxorubicin stock solutionwas added to pH-gradient liposomes (1:1 v/v)
- ✓ Doxorubicin encapsulation was determined indirectly by fluorescence assay(λex 485 nm; λem 585 nm)

$$\text{EE}\ (\%) = \frac{(\text{Feeding doxorubicine}) - (\text{Free doxorubicin})}{(\text{Feeding doxorubicin})} * 100\% \qquad (3)$$

$$\text{D/L}\ (\text{w/w}) = \frac{(\text{Feeding doxorubicin} - \text{Free doxorubicin})}{\text{Total lipid} + \text{Feeding doxorubicin}} \qquad (4)$$

- Release kinetics of doxorubicin-loaded lipoplexes:
  - ✓ doxorubicin-loaded lipoplexes was added to dialysis bags (6–8 kDa MWCO)
- Cell viability assay:
  - ✓ HeLa cells
  - ✓ After 24 h, optimized formulation were added to each well, with final concentrations of doxorubicin ranging from0.01 to 25 μM per well
  - ✓ the blank formulation (without doxorubicin)was tested for its cytotoxicity at its highest concentration
  - ✓ Absorbance was measured at 570 nm and 600 nm
  - ✓ IC50 was determined

❖ Tobramycin-loaded lipoplexes:

- ✓ Tobramycin sulphatewas fluorescently labeled by Cy5.
- ✓ The binding affinity of Apt-Ctrl-1 for Tobramycin-Cy5 was measured by fluorescence
- ✓ Liposomes (DOTAP/Chol/DSPE-PEG2000 50/48/2 molar ratio) mixed at equal volume (N/P = 3)with the tobramycin/aptamer solutions or tobramycin solutions without Apt-Ctrl-1 (negative control)
- ✓ Free Tobramycin-Cy5 was quantified in the supernatant by fluorescence using a Safire microplate reader (λexc 649 nm; λem 670 nm)
- ✓ Tobramycin encapsulation efficiency and the final drug/lipid (D/L) ratio were determined using Eqs. (3) and (4)

Results:

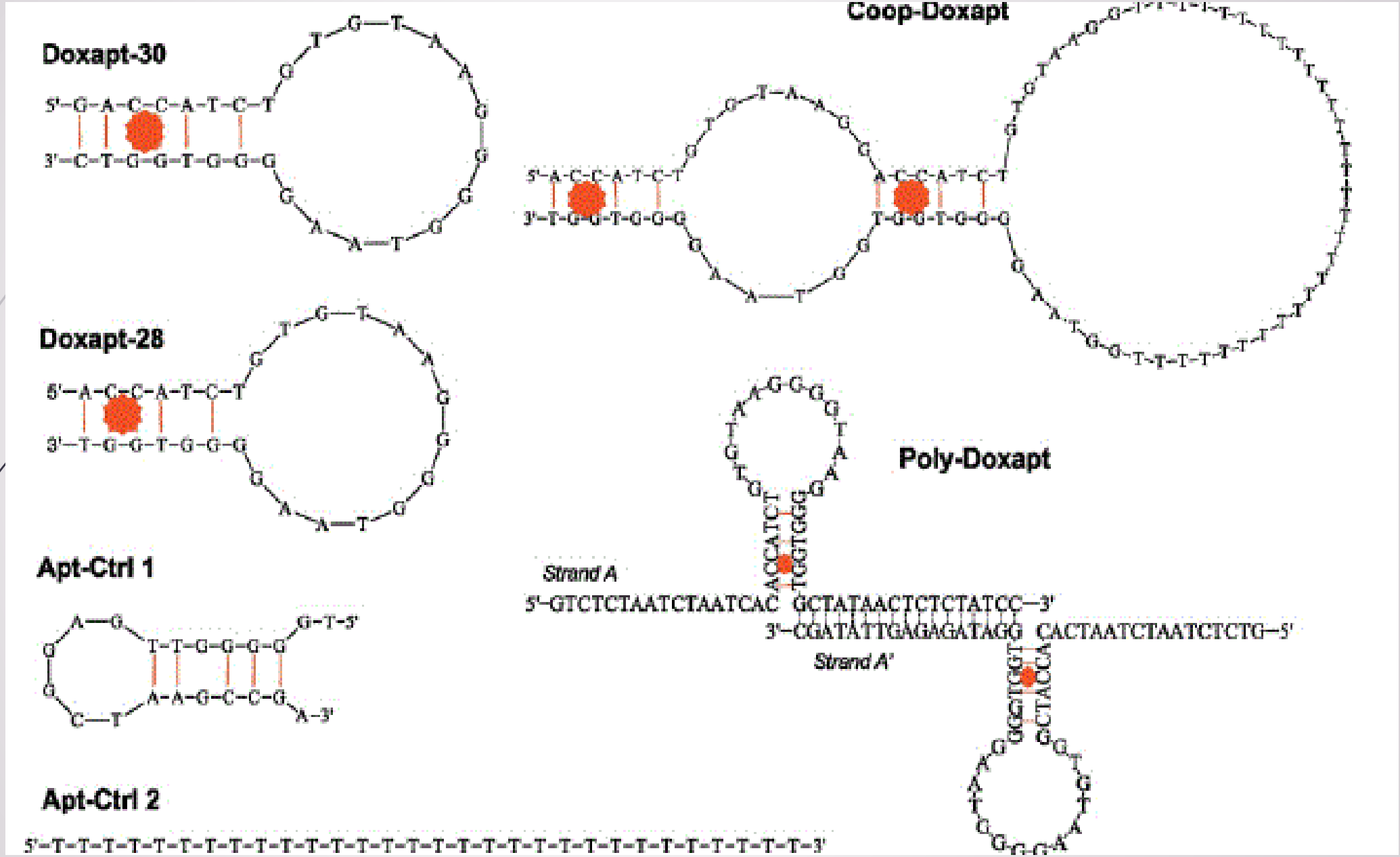
❖ Aptamer design and affinity for doxorubicin:

✓ Reasonses for selecting Doxorubicin as a model drug:

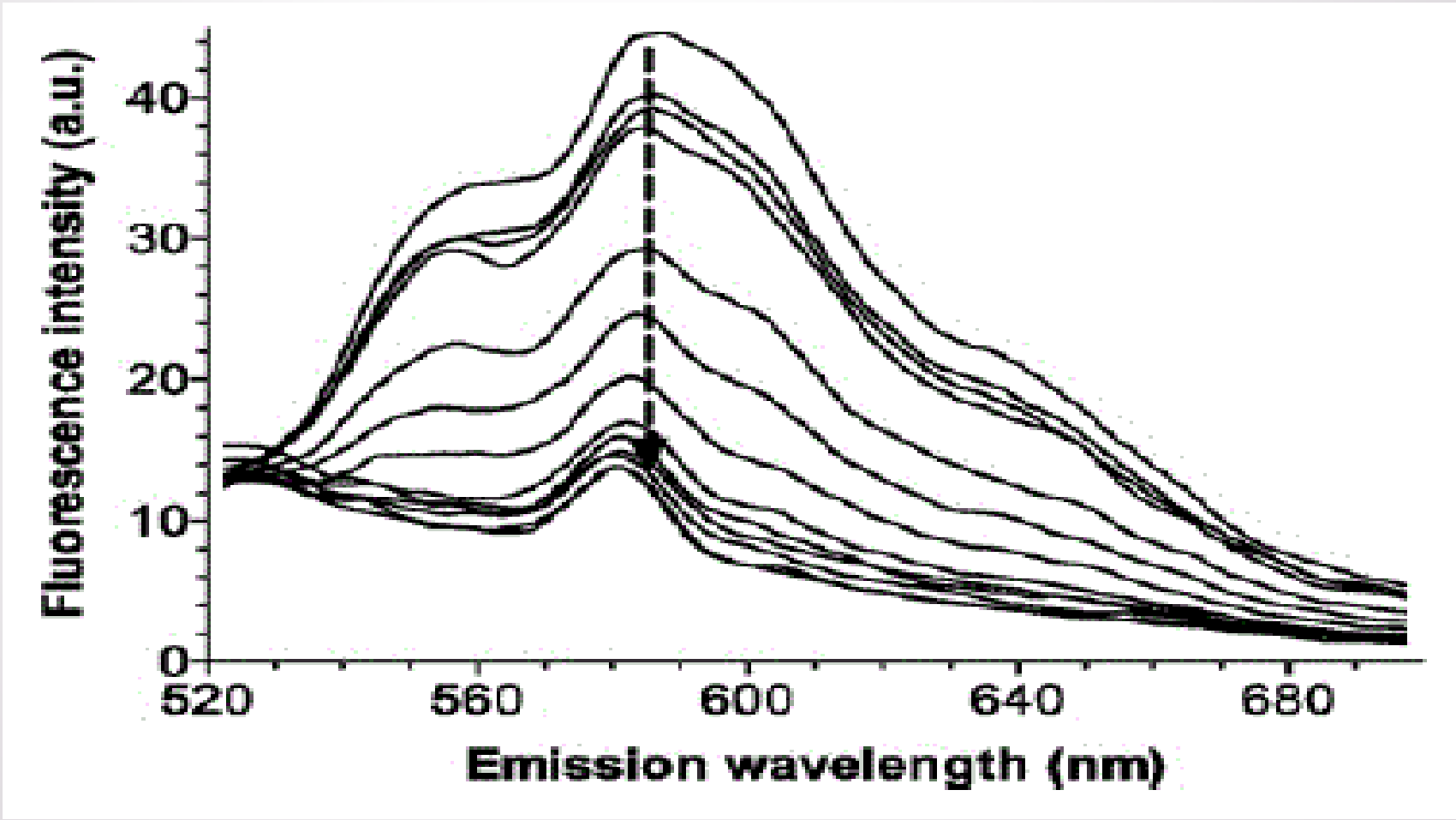
1) it represents a good example of successful active drug loading via ammoniumsulphate gradient into liposomes

2) a doxorubicin-binding DNA aptamer has already been reported

3)binding of doxorubicin to aptamers can be easily monitored by fluorescence measurements


Doxapt-30
5'-G-A-C-C-A-T-C-T
3'-C-T-G-G-T-G-G-G
Coop-Doxapt
Doxapt-28
5'-A-C-C-A-T-C-T
3'-T-G-G-T-G-G-G
Poly-Doxapt
Strand A
5'-GTCTCTAATCTAATCAC GCTATAACTCTCTATCC-3'
3'-CGATATTGAGAGATAGG CACTAATCTAATCTCTG-5'
Strand A'
Apt-Ctrl 1
G-T-5'
A-A-G-C-C-G
A-3'
Apt-Ctrl 2
5'-T-T-T-T-T-T-T-T-T-T-T-T-T-T-T-T-T-T-T-T-T-T-T-T-T-T-T-T-T-T-3'

- ✓ emission spectra of doxorubicin (100 nM in 5% dextrose and 5 mM NaCl) with increasing concentration of Poly-Doxapt (from top to bottom, 0, 0.001, 0.005, 0.01, 0.05,0.1, 0.25, 0.75, 1, 5, 10, 25 μM).



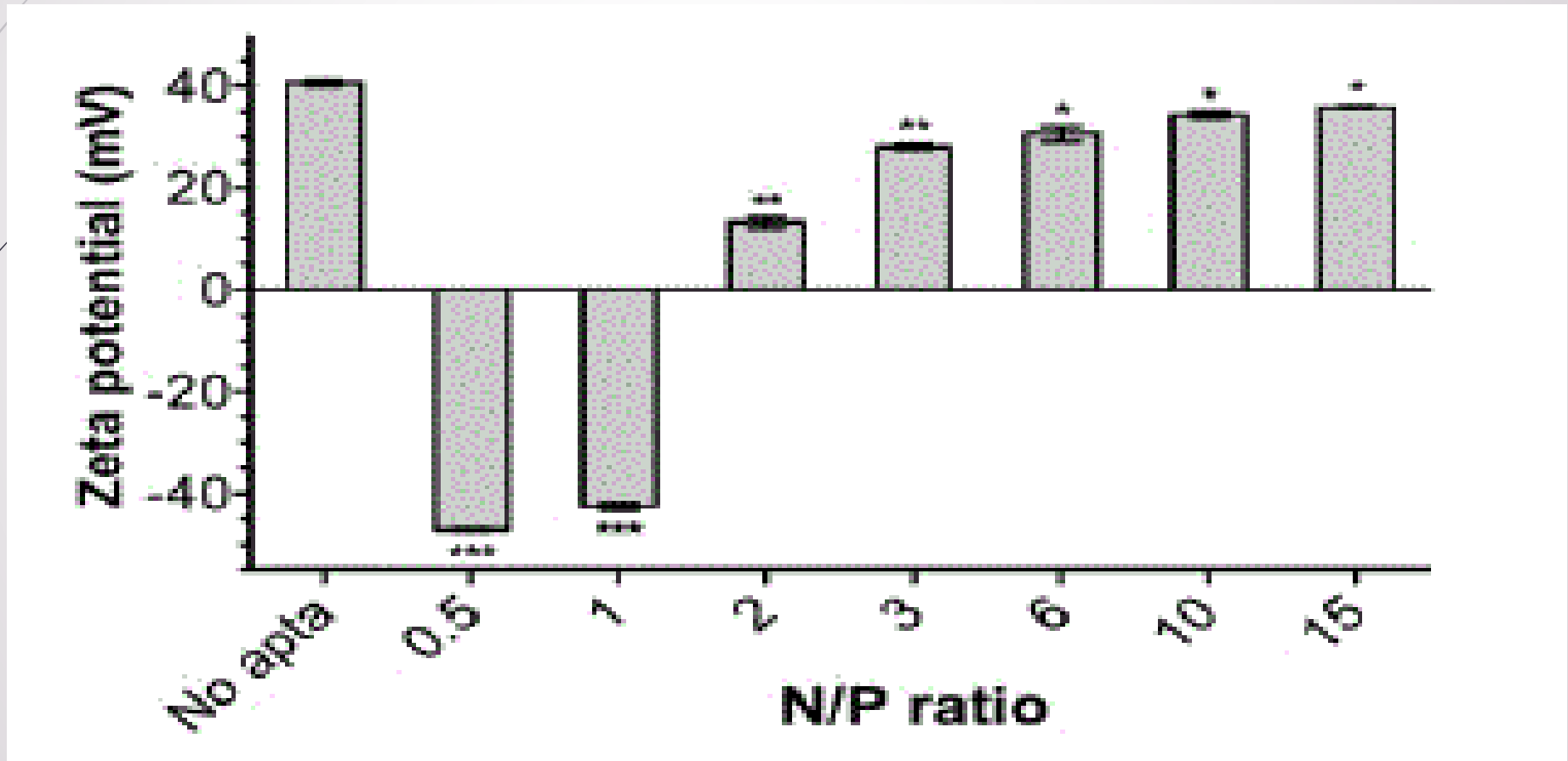
- Characteristics of aptamers used in this study and their affinity constants (KD) for doxorubicin.

Formulation	Doxapt-28	Doxapt-30	Coop-Doxapt	Poly-Doxapt	Apt-Ctrl-1	Apt-Ctrl-2
Nucleotides	28	30	86	62 + 62	21	30
Binding sites	1	1	2	2	0	0
K_D (nM)[a]	380 ± 45	334 ± 29	160 ± 36	68 ± 6	>1000	ND[b]

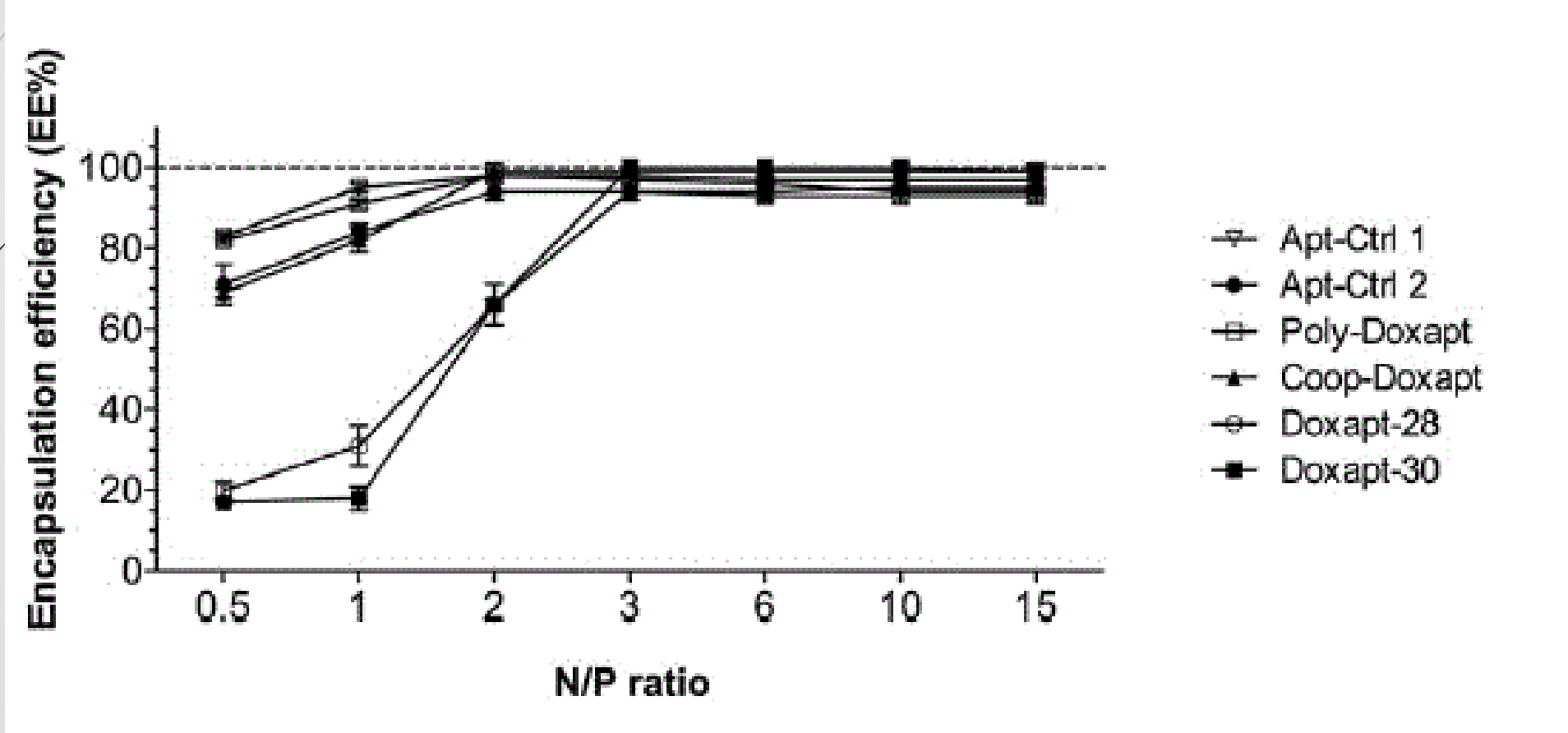
[a] Determined by fluorescence assay, as shown in Figs. 1, S1–S5.

[b] Fluorescence of doxorubicin was not quenched sufficiently to determine a dissociation constant (see Fig. S5).

- ✓ zeta potential measurements of lipoplexes encapsulating Doxapt-30:



- ✓ Encapsulation efficiency of all aptamerswithin cationic lipoplexes



- Stability of encapsulated aptamers over 24 h (Doxapt-30 (ratio N/P = 3) were assayed for their content in aptamers using the direct and indirect method

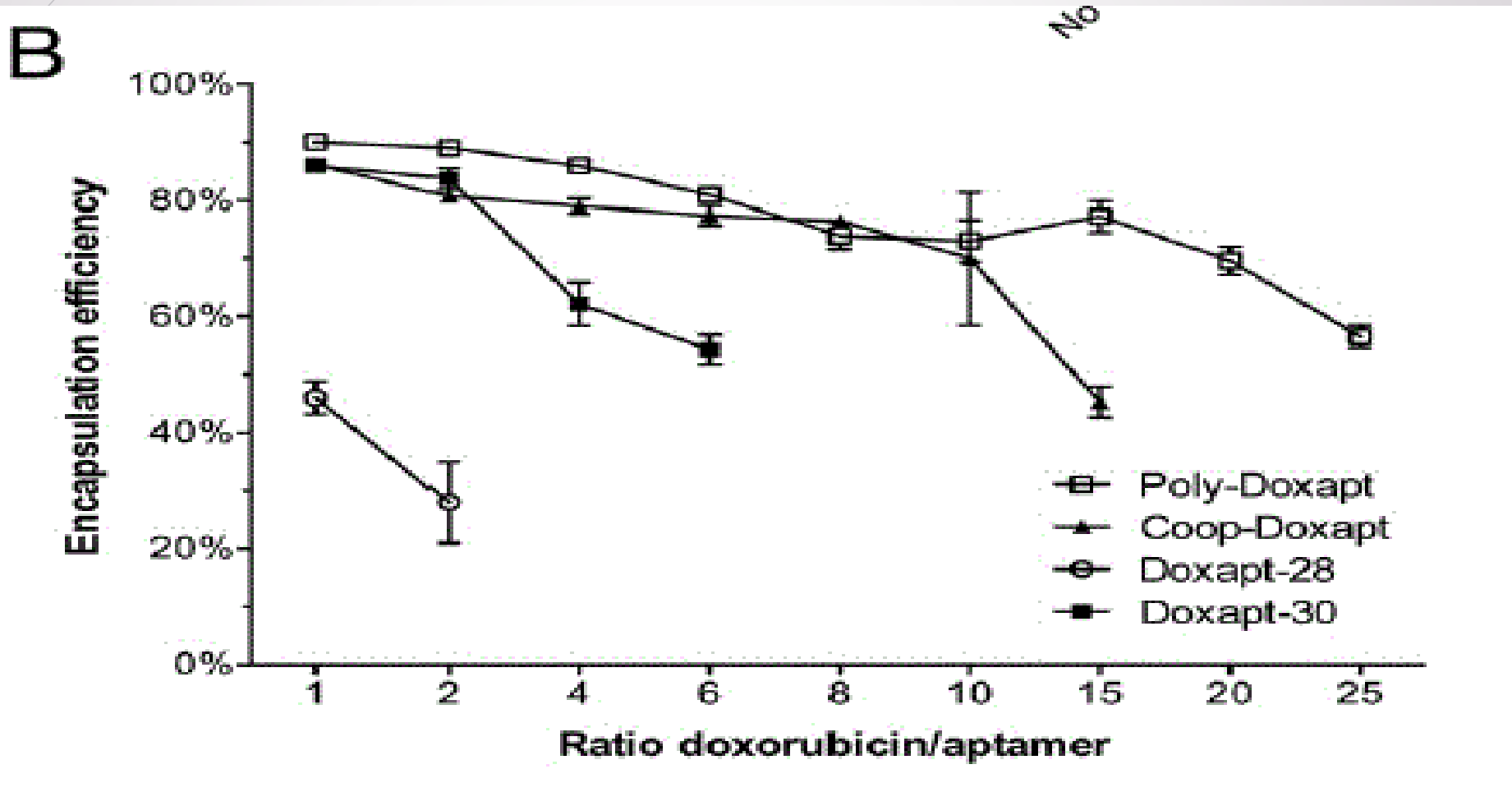
	Time (h)		
	0	4	24
Direct method	98.3 ± 0.1%	98.1 ± 0.1%	98.2 ± 0.1%
Indirect method	98.6 ± 0.6%	99.1 ± 0.3%	97.0 ± 1.2%

✓ Summary of the characteristics for the optimized formulations

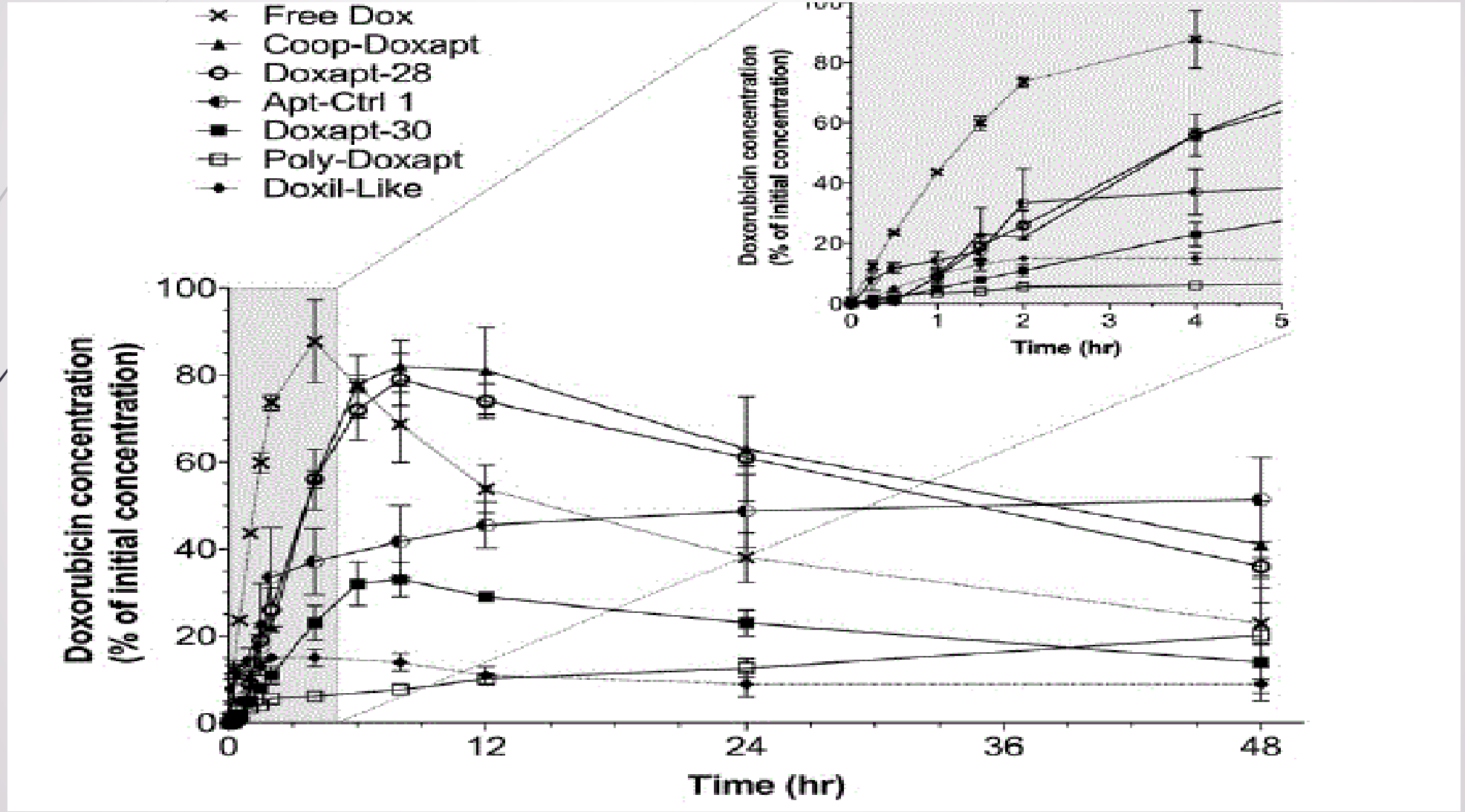
Formulation		Doxapt-28	Doxapt-30	Coop-Doxapt	Poly-Doxapt	No apta	Doxil-like	Apt-Ctrl-1	Apt-Ctrl-2
Liposomes	Z-average diameter (nm)	139 ± 3	154 ± 2	139 ± 3	139 ± 3	139 ± 3	187 ± 6	139 ± 3	139 ± 3
	PdI	0.082 ± 0.001	0.049 ± 0.012	0.082 ± 0.001	0.082 ± 0.001	0.082 ± 0.001	0.038 ± 0.036	0.082 ± 0.001	0.082 ± 0.001
Aptamer-loaded lipoplexes	Z-average diameter (nm)	171 ± 3	286 ± 2	230 ± 3	283 ± 7	139 ± 3	187 ± 6	242 ± 5	206 ± 4
	PdI	0.058 ± 0.018	0.241 ± 0.018	0.058 ± 0.019	0.222 ± 0.023	0.082 ± 0.001	0.038 ± 0.036	0.069 ± 0.013	0.048 ± 0.033
	Zeta potential (mV)	22.4 ± 1.2	28.2 ± 0.6	−19.4 ± 0.6	−24.7 ± 0.6	24.9 ± 0.6	−4.8 ± 1.1	4.53 ± 0.6	17.6 ± 1.6
	Ratio N/P	3	3	2	2	N/A	N/A	3	3
	EE% aptamers[a]	94 ± 2%	100 ± 0%	99 ± 2%	98 ± 1%	N/A	N/A	94 ± 1%	99 ± 0%
Doxorubicin and aptamer-loaded lipoplexes	Z-average diameter (nm)	197 ± 2	236 ± 6	254 ± 2	312 ± 4	154 ± 2	181 ± 3	285 ± 5	204 ± 2
	PdI	0.058 ± 0.018	0.047 ± 0.048	0.062 ± 0.002	0.172 ± 0.007	0.074 ± 0.008	0.063 ± 0.010	0.075 ± 0.011	0.068 ± 0.029
	Zeta potential (mV)	16.3 ± 0.8	15.9 ± 0.7	−28.1 ± 1.7	−28.8 ± 1.2	20.9 ± 0.9	−11.5 ± 0.1	8.81 ± 0.7	5.7 ± 0.5
	Ratio Dox/Apta	2	2	4	4	N/A	N/A	2	2
	EE% Dox[b]	28 ± 7%	84 ± 2%	73 ± 3%	86 ± 1%	5 ± 3%	98 ± 1%	39 ± 0%	7 ± 5%
	Final D/L ratio[c]	0.0034	0.0092	0.0088	0.0069	0.0006	0.098	0.0062	0.0008

[a] Determined by indirect method using Eq. (1).
[b] Determined by indirect method using Eq. (3).
[c] D/L: Drug/Lipid ratio (w/w) determined using Eq. (4).

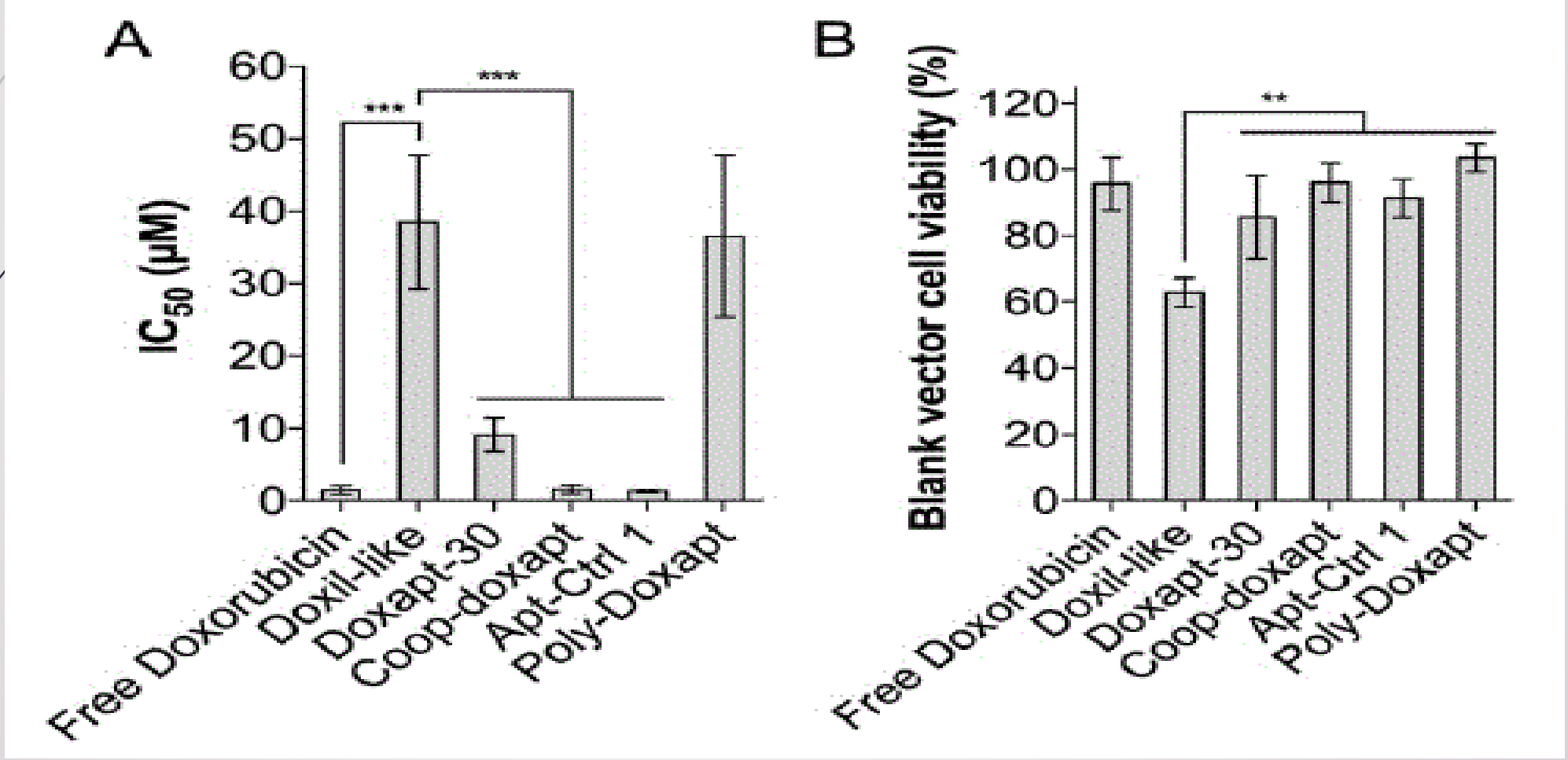
- ✓ Encapsulation efficiency of doxorubicin within all aptamer-loaded lipoplexes



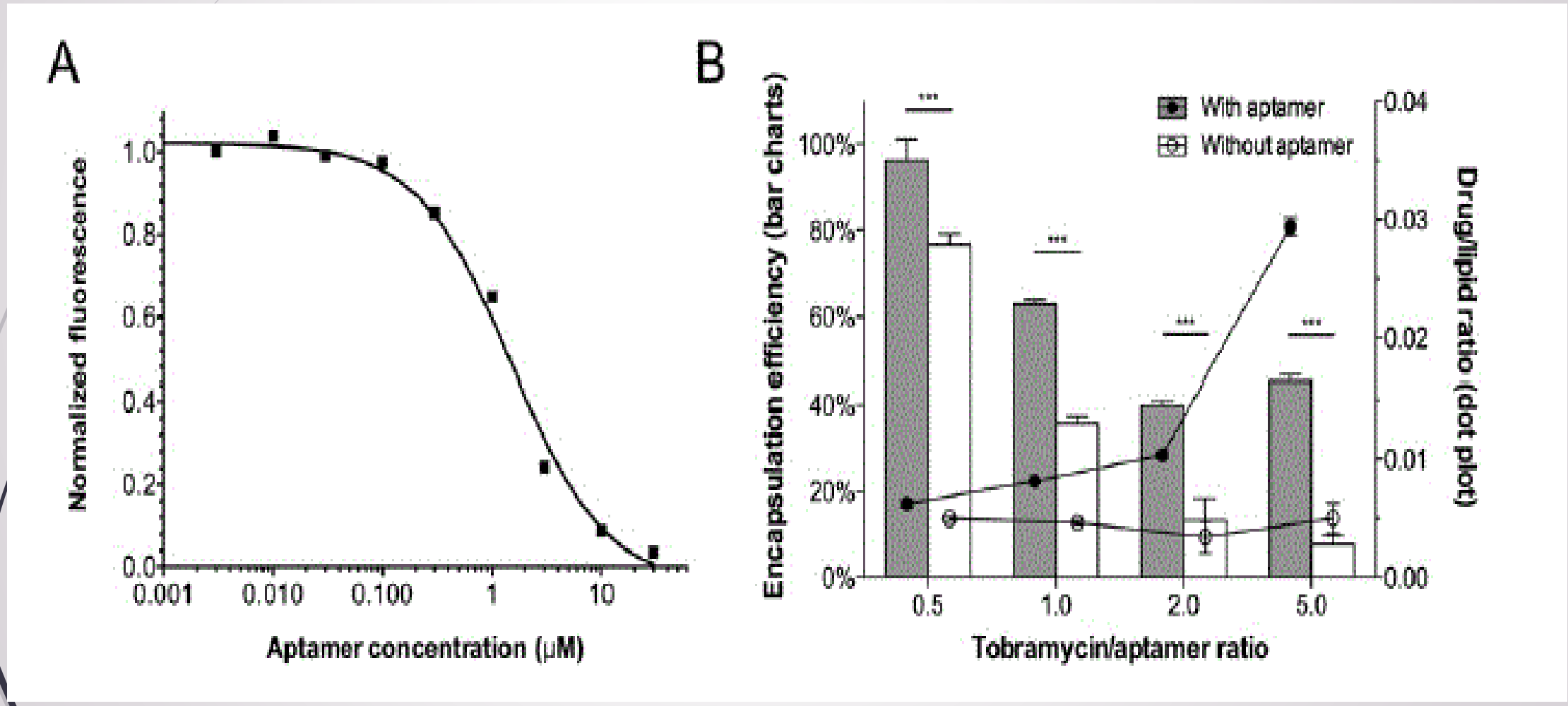
- ✓ Release of doxorubicin at 37 °C in PBS (pH 7.4) from optimized formulations of doxorubicin-loaded lipoplexes



- Cytotoxic activity of doxorubicin in optimized formulations (see Table 3) on HeLa cells(IC50 values of doxorubicin in optimized formulations after 48 h.)



✓ Tobramycin encapsulation using aptamers



Discussion:

- In the passive loading method, the drug dissolved in the aqueous phase equilibrates with the liposome's internal medium, which limits its encapsulation efficiency.
- using a gradient method, such as pH or ions, the drug,when present inside the vesicle core, is converted to an ionic/salt form, which is unable to diffuse back through the lipid membrane, thus is retained into the liposome core
- Doxil-like formulation, which exhibited limited release ,high IC50 on HeLa cells, and even a non-negligible toxicity of the liposomes itself
- Doxapt-28, presenting a similar affinity to Doxapt-30,was not able to encapsulate N50% of doxorubicin.
- non-specific binding was not detected in the binding affinity measurements ,it can be explained by the natural affinity of doxorubicin for nucleic acids or the electrostatic interactions of both cationic drugs with anionic nucleobases

- drug binding to the aptamer slowed down the release rate of the drug in comparison to the free drug, according to its affinity constant.
- Doxapt-30 exhibits a specific affinity for doxorubicin and a high encapsulation efficiency within cationic lipoplexes, which resulted in a sustained release profile and an excellent therapeutic efficiency.

Conclusion:

- ✓ Combining the advantages of aptamer specific properties and liposomal controlled release enable to achieve tunable properties according to the structure of the aptamer
- ✓ aptamers significantly improved the loading of hydrophilic tobramycin into liposomes
- ✓ adding drug specific sequences to a cancer-targeted aptamer could lead to better controlled drug delivery systems
- ✓ Further improvements are currently focused on the drug/lipid ratio and the application to larger biomacromolecules